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Remarks

Pending claims

Claims 1-132 are pending. Of these, claims 1-17, 29-49, and 61-64 are allowed; claims 18-20, 50-52, 65-83, 92-115, and 124-127 are rejected; and claims 21-28, 53-60, 84-91, 116-123, and 128-132 are withdrawn as directed to non-elected subject matter.

Amendments

The title of the specification was amended according to the Examiner's suggestion. *See*, Paper No. 8, page 4, item 2. Additionally, the Abstract was amended to delete the paragraph number and to correct minor typographical errors. No new matter was added by way of these amendments.

Restriction Requirement

Applicants acknowledge and thank the Examiner for agreeing to rejoin the subject matter of Groups II (claims 21-28, 53-60, 84-91, and 116-123) and III (claims 128-132) with the product claims of Group I, should the claims of Group I be found allowable. *See*, Paper No. 8, page 4, second paragraph.

Claim rejection under 35 U.S.C. § 101

Claims 65-83, and 124-127 were rejected under 35 U.S.C. § 101 as allegedly directed to non-statutory subject matter. *See*, Paper No. 8, page 4, item 3. In particular, it was asserted that "[t]he claims embrace an antibody as it occurs *in vivo* in the immunized animal. However, . . . amending the claims to require the hand-of-man would obviate this rejection" *See*, Paper No. 8, pages 4-5, item 3.

Applicants respectfully disagree and traverse.

Independent claim 65 and dependent claims 66-83 cite "[a]n isolated antibody or portion thereof" (emphasis added). This language alone specifies that the claimed antibody or portion thereof is the product of human intervention. Furthermore, the language of independent claim 124, and dependent claims 125-127, also indicates that the claimed antibody or portion thereof is the product of human intervention, as the antibody is made by a process that requires the immunization of an animal. Therefore, Applicants assert that the claims do not embrace a naturally occurring antibody. Applicants respectfully request that

the rejection of claims 65-83, and 124-127 under 35 U.S.C. § 101 be reconsidered and withdrawn. Should the Examiner disagree with the above explanation, Applicants' representative would welcome the opportunity to discuss this issue with the Examiner by telephone in order to reach a mutually acceptable agreement.

Claim rejection under 35 U.S.C. § 112, 1st paragraph

Claims 65-83, 92-115, and 124-127 were rejected under 35 U.S.C. § 112, first paragraph. *See*, Paper No. 8, pages 5-6, item 4. In particular, the Examiner has requested an affidavit or declaration pertaining to ATCC Deposit 97157. *See*, Paper No. 8, page 5, last full paragraph.

Accordingly, Applicants herein submit a declaration regarding ATCC Deposit number 97157, which was made under the terms of the Budapest Treaty.

> Human Genome Sciences, Inc., the assignee of the present application, has deposited biological material under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209 (present address). The deposit was made on May 22, 1995, accepted by the ATCC, and given ATCC Accession Number 97157. In accordance with M.P.E.P. § 2410.01 and 37 C.F.R. § 1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC Accession Number 97157 will be irrevocably removed upon the grant of a patent based on the instant application, except as permitted under 37 C.F.R. § 1.808(b). A partially redacted copy of the ATCC Deposit Receipt for Accession Number 97157 is enclosed herewith as Exhibit A.

In view of the above, Applicants believe the Examiner's concerns have been fully addressed. Accordingly, Applicants respectfully request that the rejection of claims 65-83, 92-115 and 124-127 be reconsidered and withdrawn.

Claim rejection under 35 U.S.C. § 112, 2nd paragraph

Claims 18-20, 50-52, 81-83, and 113-115 were rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite for failing to particularly point out and distinctly

claim the subject matter which applicant regards as the invention. *See*, Paper No. 8, page 6, item 5. In particular it was asserted that:

> Claims 18-20, 50-52, 81-83, and 113-115 are drawn to a hybridoma that produces monoclonal antibodies to the protein of amino acid sequence set forth in SEQ ID NO:2. It is suggested that Applicants amend these claims to depend on claims 11, 35, 67, and 98 (drawn to monoclonal antibodies) respectively, rather than on the independent claims they depend from, since only hybridomas produce monoclonal antibodies."

See, Paper No. 8, page 6, item 5 (emphasis added).

Applicants respectfully disagree and traverse.

Claims 18, 50, 81, and 113 are drawn to "[a]n isolated cell" and are not limited to hybridoma cells. When the present application was filed it was well known in the art that cells other than hybridomas could be used to produce antibody molecules. For example, Applicants submit herewith Exhibit B, a 1994 review article[1] which describes expression of antibody molecules on bacteriophage (grown in bacterial cells). As another example, the present specification at paragraph [0120] directs the reader to U.S. Patent 4,946,778, which describes production of single chain antibody molecules in mammalian, yeast, fungal, and bacterial cells. Additionally, Applicants also submit herewith Exhibit C, an excerpt from a 1994 immunology text book[2] which describes production and selection of bacteriophage *monoclonal* antibody molecules. Hence, Applicants respectfully note that the assertion that "only hybridomas produce monoclonal antibodies" is inaccurate. Conversely, hybridoma cells are also not limited to production of monoclonal antibodies. For example, Applicants submit herewith Exhibit D with abstracts from three publications[3] describing hybridomas that produce bi-specific and polyclonal antibodies. (Applicants will submit a copy of the complete articles at the Examiner's request.) In sum, Applicants respectfully submit that the dependencies of claims 18-20, 50-52, 81-83, and 113-115 are properly drawn and request that

[1] Winter, et al., "Making Antibodies by Phage Display Technology," Annu. Rev. Immunol., 12:433-55 (1994).
[2] Janeway, et al., "Immunobiology: The Immune System in Health and Disease," Current Biology Ltd., Garland Publishing, New York, NY, pp. 2:23-2:25 (1994).
[3] Thompson, et al., "Production of human monoclonal IgG and IgM antibodies with anti-D (rhesus) specificity using heterohybridomas," Immunol., 58:157-160 (1986); Matsuo, et al., "Generation of multiple monoclonal antibodies for diagnostic use from a single hybridoma fusion," Acta. Haematol., 179:137-139 (1988); and,

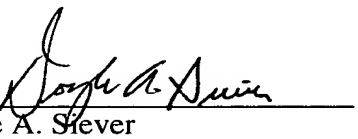
the rejection of these claims under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn. Should the Examiner disagree with the above explanations, Applicants' representative would welcome the opportunity to discuss this issue with the Examiner by telephone.

Conclusion

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Dated: May 19, 2003.

Respectfully submitted,

By 

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MMW/LJH/DAS/FR

VIA HAND DELIVERY MAY 21, 2003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Ni et al.

Docket No.: PF199D2

Application No.: 09/911,346- Conf: 4955

Group Art Unit: 1646

Filed: July 24, 2001

Examiner: P. Mertz

For: **Antibodies to Natural Killer Cell Enhancing Factor C** (As Amended Herein)

VERSION WITH MARKINGS TO SHOW CHANGES MADE

(Underline indicates text inserted, Strike-through indicates text deleted)

In the specification

The Title was amended to read:

Antibodies to Natural Killer Cell Enhancing Factor C

The Abstract was amended to read:

~~[0154]~~ A human natural killer cell enhancing factor C and fragments thereof and DNA (RNA) encoding such polypeptides and a procedure for producing such [~~polypeptidees~~] polypeptides by recombinant techniques is disclosed. Further disclosed are antibodies directed against such polypeptides and fragments or portions thereof and methods for producing such antibodies and utilizing such antibodies for therapeutic or diagnostic purposes. Also disclosed are methods for utilizing such polypeptides and/or antibodies for preventing and/or treating viral infections, inflammation, neoplasia and [~~damge~~] damage from superoxide radicals. Diagnostic assays for identifying mutations in nucleic acid sequence encoding a polypeptide of the present invention and for detecting altered levels of the polypeptide of the present invention for detecting diseases, for example, cancer, are also disclosed.

American Type Culture Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attention: Robert H. Benson
9410 Key West Avenue
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc. - Docket PF202 and PF199

Identification Reference by Depositor:	ATCC Designation
DNA Plasmid, 652259	97157 PF199

The deposits were accompanied by: _ a scientific description _ a proposed taxonomic description indicated above.

The deposits were received May 22, 1995 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested June 6, 1995. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Annette L. Bade, Director, Patent Depository

Date: June 6, 1995

cc: Greg D. Ferraro, J.D.

BP4/9

Annu. Rev. Immunol. 1994. 12:433–55

MAKING ANTIBODIES BY PHAGE DISPLAY TECHNOLOGY

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KEY WORDS: selection, repertoires, rearranged V-genes, V-gene segments, polymerase chain reaction

Abstract

Antibody fragments of predetermined binding specificity have recently been constructed from repertoires of antibody V genes, bypassing hybridoma technology and even immunization. The V gene repertoires are harvested from populations of lymphocytes, or assembled in vitro, and cloned for display of associated heavy and light chain variable domains on the surface of filamentous bacteriophage. Rare phage are selected from the repertoire by binding to antigen; soluble antibody fragments are expressed from infected bacteria; and the affinity of binding of selected antibodies is improved by mutation. The process mimics immune selection, and antibodies with many different binding specificities have been isolated from the same phage repertoire. Thus human antibody fragments have been isolated with specificities against both foreign and self antigens, including haptens, carbohydrates, secreted and cell surface proteins, viral coat proteins, and intracellular antigens from the lumen of the endoplasmic reticulum and the nucleus. Such antibodies have potential as reagents for research and in therapy.

INTRODUCTION

In the immune system, the rearrangement of the V gene segments creates a repertoire of virgin B cells, each displaying a single antibody species.

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Cells are selected by encounter and binding of antigen, and they are triggered to differentiate to short-lived plasma cells that secrete antibody and to long-lived memory cells that persist in lymph nodes, spleen, and bone marrow. The V genes of the selected antibodies displayed on memory cells are subject to hypermutation, leading to antibodies of improved binding affinity after further selection with antigen. Thus repeated immunization leads to "affinity maturation" of the response (Figure 1). The immortalization of antigen-stimulated B cells by fusion to myeloma cells (1) taps the immune repertoire and has led to a wealth of rodent monoclonal antibodies with predefined specificity.

Technologies have been emerging for making antibodies in vitro by mimicking the selection strategies of the immune system (2–4). Repertoires of antibody fragments are displayed on the surface of filamentous bacteriophage, each displaying a single antibody species; the phage are selected by binding to antigen; and finally soluble antibody fragments are secreted from infected bacteria (Figure 1). As in the immune system, the V genes can be subjected to random mutation, and mutants may be selected with higher binding affinities. This allows the isolation of human antibody fragments of defined specificity, against both foreign and self-antigens. The technology is evolving fast (reviewed in 5–7), and here we review recent progress.

TECHNOLOGIES FOR SELECTION

Mimicking the B Cell

In the immune system, the B cell represents a self-replicating package containing the antibody genes that encode the antibody displayed at its surface. Phage display mimicks the B cell. Filamentous phage was first used to display small peptides by fusion to the minor coat protein (pIII: probably three or five copies per phage particle; here illustrated with three copies) (8). Two sites of pIII were used for fusion: in the flexible spacer between the two domains of pIII (8), or close to the N-terminus (9) or at the N-terminus (10). The phage were enriched by binding of peptide to monoclonal antibody. Through growth of the enriched phage and further selection by binding to antibody, very rare phage could be isolated (8).

Surprisingly, folded antibody fragments (2) and other proteins (11, 12) can also be displayed on phage. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer (13, 14), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the periplasm (2, 15–18). When antibody fragments are fused to the N-terminus of pIII, the phage is infective (2, 15). However, if the

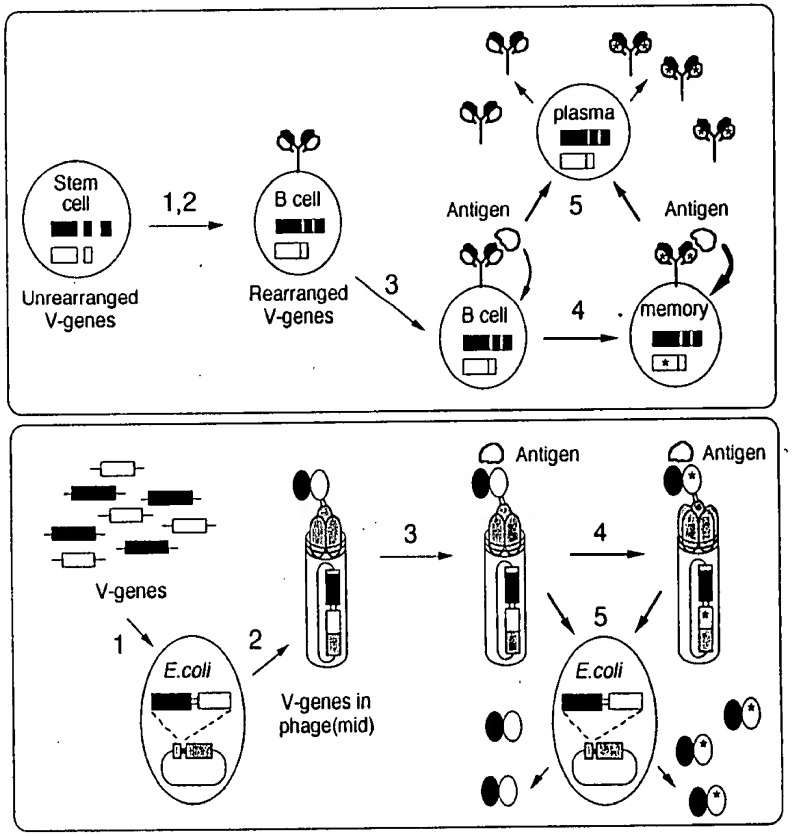

Figure 1 Generation of antibodies by the immune system and phage technology. Steps: (1) rearrangement or assembly of germline V genes; (2) surface display of antibody (fragment); (3) antigen-driven or affinity selection; (4) affinity maturation; (5) production of soluble antibody (fragment).

N-terminal domain of pIII is excised and fusions made to the second domain, the phage is not infective, and wild type pIII must be provided by helper phage (see below) (11, 16, 17) (Figure 2).

The pIII fusion and other proteins of the phage can be encoded entirely within the same phage replicon (2, 8), or on different replicons (11, 15–19). When two replicons are used, the pIII fusion is encoded on a phagemid, a plasmid containing a phage origin of replication. Phagemids can be packaged into phage particles by "rescue" with a helper phage such as

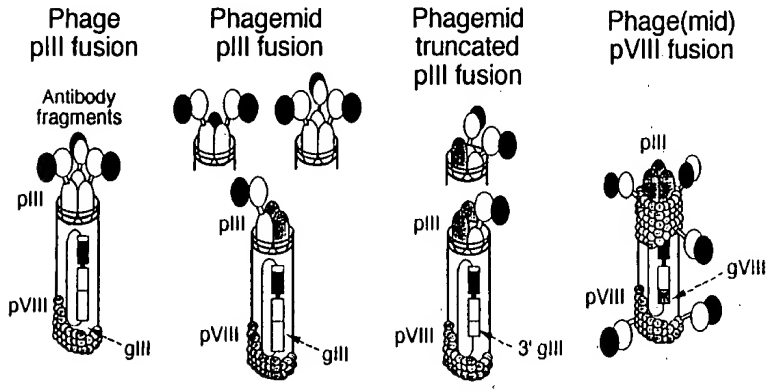

Figure 2 Display of antibody domains as pIII and pVIII fusions using phage and phagemid vectors. Antibody domains are depicted as black (heavy chain, VH or VHCH1) or white (light chain, VL or VLCL) spheroids; the genes are marked in similar fashion. Depicted are pIII fusion as phage (2) or phagemid (15) (18); truncated pIII fusion as phagemid (16, 17); pVIII fusion as phage (28, 30) or phagemid (27, 29). Only infectious phage particles displaying antibody domains are shown.

M13K07 that provides all the phage proteins, including pIII, but due to a defective origin is itself poorly packaged in competition with the phagemids (20).

The pIII fusion is often proteolysed, as shown by gel electrophoresis of the phage proteins and detection with anti-pIII antisera (J McCafferty, unpublished data). This is expected to give a population of phage particles, each displaying zero, one, two, three (and perhaps four and five) antibody fragments. The average valency of the population is further reduced by use of helper phage, in which the helper pIII competes for incorporation into the phage particle. Such phage have been estimated on average to display less than a single fusion protein per particle; they have been termed "monovalent" phage (17, 21). Other helper phages (M13ΔgIII) that lack pIII have been designed to rescue phage particles that incorporate only the pIII fusion from the phagemid; these are therefore multivalent (22). Use of different helpers can thereby alter the valency of the phages.

The major coat protein of the phage (pVIII: 3000 copies per phage particle) can also be used to display peptides (23–26) and antibody fragments (27–30). Pentapeptides (23, 24) and hexapeptides (25) were fused close to the N-terminus of pVIII, but phage encoding longer peptides were not viable unless wild type pVIII was provided (25, 26). The phage population is multivalent. With helper pVIII, up to about 900 peptides (25) and 24 antibody fragments (27) are incorporated per phage particle.

Fusions to pIII rather than pVIII have to date been preferred for antibody display.

Mimicking Immune Selection

In the immune system, encounter with antigen involves triggering the B cell through its receptor, and proliferation and differentiation to produce plasma cells that secrete antibody (reviewed in 31). The process appears capable of selecting one or more B cells from repertoires of $< 5 \times 10^8$ cells in mice and $< 10^{12}$ cells in humans (for review, see 32). Furthermore the immune system is able to selectively enrich for B cells displaying antibodies with slightly improved binding affinities, allowing affinities to be built up in a step-wise manner through rounds of mutation and selection (33).

Phage selection appears to be at least as powerful as immune selection. Phage displaying small peptides can be selected by direct binding to solid phase antibody (8), and also by binding to a biotinylated antibody in solution, which is then captured onto solid phase streptavidin (9). Likewise phages displaying antibodies can be selected by binding to antigen coated plates (16, 34), column matrices (2), cells (35), or to biotinylated antigen in solution followed by capture (36). The phages bound to the solid phase are washed and then eluted by soluble hapten (37), acid (16) or alkali (34). Phages can be enriched 20–1000 fold by a single round of selection (2, 17, 34). Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection. In this way, enrichment factors of only 50-fold in each round can build up to 10^7-fold enrichments over four rounds of selection (34).

SELECTION EFFICIENCY The efficiency of selection is likely to depend on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with (solid phase) antigen. For example, antibodies with fast dissociation kinetics (and weak binding affinities) should be retained by use of short washes, multivalent display and a high coating density of antigen at the solid phase. The high density should not only stabilize the phage through multivalent interactions, but favor rebinding of phage that has dissociated. Nevertheless, it appears that binding affinities (for a single antibody fragment) of $10^5\ M^{-1}$ are barely sufficient to hold multivalent phage to solid phase (37).

Conversely the selection of antibodies with slow dissociation kinetics (and good binding affinities) should be promoted by use of long washes (11), monovalent phages (11), and a low coating density of antigen (38). In principle, phages with very high affinities ($> 10^{10}\ M^{-1}$) should be difficult to elute, but a change in pH may suffice to dissociate the complex (21, 39);

the phage also survive 5M guanidine hydrochloride (M Figini, unpublished data).

DISCRIMINATION In immune selection, the virgin B cells displaying antibodies with (unwanted) self-specificities are deleted or rendered anergic (40). With phage, it has proved more difficult to deplete the repertoire, for example by preabsorption, as it is difficult to capture all the phage that can bind, and many of the phages are "bald," lacking antibody fragments due to proteolysis. Nevertheless, preabsorption (on red blood cells lacking the blood group E antigen) was used for isolation of phage specificities against the blood group E antigen (35).

As with immune selection, it is also possible to select between phage antibodies of different affinities (37), even with affinities that differ slightly (36). In the later immune response, B cells are thought to compete for limiting antigen in the germinal centres (for review, see 41). Likewise in selection of peptide phages with biotinylated antibody (9), limiting antibody was used to promote competition between the phages (42).

However, random mutation of a selected antibody is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting antigen, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess soluble biotinylated antigen, but with the antigen at a lower concentration than the target affinity constant. The phages are then captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, provided no two antibody fragments on the same phage bind to the same molecule of antigen. Using this technique, mutant phage antibodies have been selected from a great excess of phages with two- to four-fold lower affinities over many rounds of selection (36, 112).

Discrimination can be enhanced by taking advantage of dissociation kinetics. Thus for two phages dissociating from antigen with slightly different kinetics, the discrimination should increase with time due to the exponential nature of the decay. Indeed this was demonstrated by dissociation of phages from biotinylated antigen in solution (36). Using such kinetic selection, even mutant antibodies with a two-fold higher affinity could be selected from a great excess of phages with lower affinity (112). Washing of phages bound to a solid phase should also discriminate by dissociation kinetics.

Discrimination may be compromised by multivalent interactions (10, 11, 16, 21), but this will depend on the affinities, kinetics, and the selection process. Multiple interactions increase the avidity of phage binding, and slight differences in affinity between two antibodies should in fact give rise to greater differences in avidity between the two phages, potentially enhancing discrimination. However, if the avidities became so strong that both phages bound very tightly to the solid phase antigen, discrimination would be lost, especially with low stringency washes.

Mimicking the Plasma Cell

Antibody fragments can be characterized and used as free soluble fragments or as phage. Binding can be detected by ELISA using antisera against the phage (2); the affinity of binding can be measured with soluble radioactive antigen (17); and dissociation kinetics by loss of phage from its complex with biotinylated antigen (36, 112). Furthermore, phages displaying antibody fragments can be used as reagents in Western blots, and for fluorescence staining of cells (A Nissim, unpublished data).

Phagemid vectors can also be engineered for display or for secretion of free antibody fragments from infected bacteria. By incorporating an amber stop codon between the fragment and pIII, the antibody fragments are fused to pIII and displayed when the amber codon is suppressed, and secreted when it is not (15). The growth of phage in suppressor and nonsuppressor bacteria therefore mimics respectively the surface display of antibodies on B cells, and the production of fragments from plasma cells (Figure 3). The same approach was used for display and secretion of human growth hormone (21). Less conveniently, the V genes encoding antibody fragments can be recloned for secretion (16, 37).

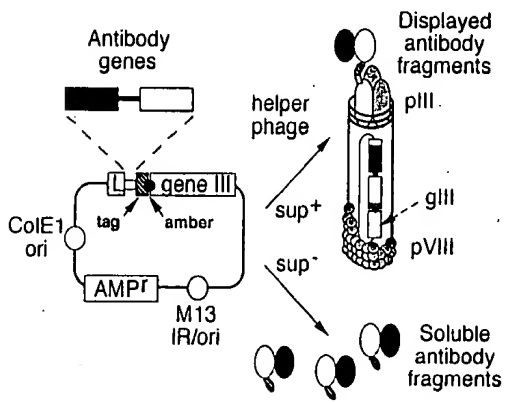

Figure 3 Mimicking the plasma cell. Phagemid pHEN1 (15) allows antibody domains to be displayed on phage after rescue with helper phage from an E. coli suppressor strain, or the domains to be secreted as (tagged) soluble fragments from non-suppressor strains. AMP = ampicillin resistance gene, L = leader peptide sequence, tag = c-myc peptide sequence.

Antibody fragments can be secreted from bacteria with yields ranging from 0.2–2 mg/l fragments in shaker flasks (43–45), or >500 mg/l in fermenters (46); and they can be harvested from the culture supernatant (44) or the periplasm (43). Protein A has been used to purify antibody fragments of the human VHIII family (47), and protein G to purify Fab fragments by binding to the CH1 domain (48). Engineered C- or N-terminal peptide tags that bind to monoclonal antibodies (49, 50) or to streptavidin (51) have also been used for both purification and detection of antibody fragments, but hexahistidine tags binding to immobilized metal chelate groups (52) seem particularly valuable for purification (53).

Antibody fragments can be characterized on a solid phase or in solution. Attempts have been made to measure binding affinities by competition ELISA (54), but the method is only qualitative (55) and may be more suitable for ranking of binding affinities. Even so this assumes no aggregation or dimerization. Thus, the reported in vitro affinity maturation of antibody fragments (56) could have been due to dimerization of the scFv fragments (57, 58). A more rigorous ELISA method (59) based on equilibrium capture would have been more suitable (55). Antibody fragments have also been characterized by binding to an antigen-coated surface by surface plasmon resonance (38, 57, 60). However, account needs to be taken of the fraction of active antibody (for determination of association rates), and of dimerization and of rebinding to the highly coated surface (for determination of dissociation rates) (61). For measurement of affinities in solution, the use of fluorescence quench titrations is often suitable for haptens (37, 62), but it is more difficult for protein antigens unless there is a large quench on binding (63).

TECHNOLOGIES FOR MAKING V-GENE REPERTOIRES

Diversity of Antibody Sequences and Structure

In the immune system the sequence diversity of antibody binding sites is not encoded directly in the germline but is assembled in a combinatorial manner from V gene segments. In human heavy chains, the first two hypervariable loops (H1 and H2) are drawn from <50 VH gene segments (64), which are combined with D segments and JH segments (65) to create the third hypervariable loop (H3). This loop is exceptionally variable in sequence and length (2–26 residues) (66); because the joining of the segments is imprecise, different reading frames of the D segment may be used, nucleotides can be inserted and deleted at the junctions, and the D segments can recombine as D-D fusions (67).

In human light chains, the first two hypervariable loops (L1 and L2) and much of the third (L3) are drawn from probably <30 Vλ (68) and <30 Vκ gene segments (JPL Cox, IM Tomlinson, unpublished data). These segments are combined with Jλ and Jκ segments to complete the third hypervariable loop (L3). This loop has limited variability. It ranges in size from 7 to 11 residues in λ light chains (69) and is most commonly 6 residues in κ light chains (70) but can vary between 5 and 8 residues (71). Thus, most of the sequence diversity (and structural diversity—see below) is encoded by the heavy chains.

Despite the immense sequence diversity, most of the loop conformations of antibody binding sites are relatively conserved (72–74). Implicit in the sequences of the VH germline segments are three major conformations for the H1 loop and five for the H2 loop. In combination they provide seven different folds (74). By contrast, the H3 loop of the rearranged heavy chains is likely to provide a huge range of structures. Implicit in the sequences of the Vλ segments are at least three major conformations for the L1 loop and at least two for the L2 loop (68). In the Vκ segments, there are probably four major conformations for the L1 loop and one for the L2 loop; in combination these provide four different folds (JPL Cox, IM Tomlinson, unpublished data). The combinations of different loops, decorated with side chains, create a wealth of binding sites ranging from flat surfaces (75) to pockets (76).

The potential diversity of different sequences in the primary immune repertoire is far greater than the number of B cells at any time. However, some sequences may not fold, and others may produce identical loop conformations: the repertoire of binding site structures is likely therefore to be much smaller than the sequence repertoire. Presumably the V gene segments and their representation in the expressed antibody repertoire reflect the efforts of the immune system over evolution to encode a diverse structural repertoire with a limited number of B cells. For phage repertoires, the V gene segments appear therefore to be suitable building blocks for making a diverse repertoire of structures.

Repertoires of VH and VL genes

The use of the polymerase chain reaction, with primers matching the 5′ and 3′ ends of rearranged VH and VL genes, provided the means to amplify, clone, and express V genes from lymphocytes (77), thereby making diverse V gene repertoires for expression (Figure 4). The V genes may be amplified from both cDNA and genomic DNA, with back primers at the 5′ end of the exon encoding the mature V-domain and forward primers based within the J-segment (49, 77). However, for amplifying from cDNA, "back" primers have also been based in the leader exon (79), and forward primers within the constant region (78). To maximize complementarity,

degeneracy was incorporated into the primers (77, 78), or different primers were designed for different families of V genes (80). For cloning of the amplified DNA into expression vectors, rare restriction sites were introduced within the PCR primer (77), as a "tag" at one end, or by further PCR amplification with a tagged primer (37). "Primary" repertoires of V genes harvested from a lymphocyte population are likely to contain somatic mutations, although most published human VH and Vκ gene sequences encode few (<5) amino acid substitutions (64; JPL Cox, IM Tomlinson, unpublished data).

Repertoires of "synthetic" rearranged V genes have also been derived in vitro from V gene segments (Figure 4). Most of the human VH-gene segments have now been cloned, sequenced (64), and mapped (81); these cloned segments (including all the major conformations of the H1 and H2 loop) have been used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length (47; A Nissim, unpublished data). VH repertoires have also been made with all the sequence diversity focussed in a long H3 loop of a single length (82). Human Vκ and Vλ segments have been cloned and sequenced (68; JPL Cox, IM Tomlinson, unpublished) and are therefore available for making synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, should encode antibodies of considerable structural diversity.

Combining VH and VL Gene Repertoires

Most of the structural diversity of antibody binding sites appears to be contributed by heavy rather than light chains (see above). Indeed, heavy

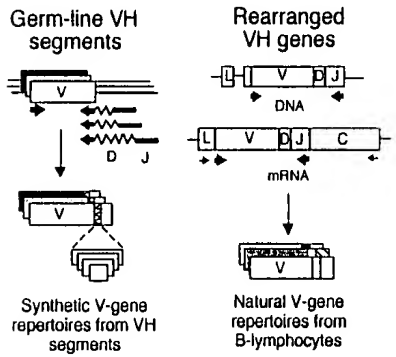

Figure 4 Generation of V gene repertoires. The location of the primers for PCR amplification of V gene repertoires from segments (47) or from rearranged V genes (34, 37) are indicated.

chains and VH domains (49) have been found with binding activities in the absence of the light chain. Furthermore in camels, two of the heavy chain isotypes lack the CH1 domain and do not appear to associate with light chains (83). However, the structures of complexes of antibody and antigen indicate that usually both domains make important interactions (75, 84–86). Presumably the role of VL domains is to add structural diversity, for example, in helping to make binding clefts, and to create a larger surface of interaction with antigen. Both features should enhance the probability of finding an antibody that binds to antigen with good affinity.

Repertoires of antibody fragments have been constructed by combining VH and VL gene repertoires together in several ways (Figure 5). Each repertoire can be created in different vectors, and the vectors recombined in vitro (87) or in vivo (88); alternatively, the repertoires may be cloned sequentially into the same vector (16) or assembled together by PCR and then cloned (37). A technique of "in-cell PCR assembly" has also been described for combining the VH and VL genes within the lymphocyte by PCR, and then cloning the repertoires of linked genes (89). Repertoires of VH domains have also been combined with a single VL gene (47, 82). The route by which repertoires are combined can dictate the structural diversity and repertoire size. For example, combining VH and VL repertoires in vivo, by combinatorial infection (88) (see below), should allow the creation of libraries of $>10^{12}$ different VH/VL combinations.

ANTIBODIES MADE FROM PHAGE DISPLAY

Taking Advantage of Immunization

Immunization leads to an increase in the number of cells making an immune response, but especially in the levels of mRNA. Resting B cells make about 100 copies of Ig mRNA per cell, whereas a hybridoma (and also presumably a plasma cell) makes about 30,000 copies (90). Spleen, lymph nodes, tonsils, and bone marrow (but not peripheral blood lymphocytes) provide a rich source of plasma cells and Ig mRNA. Repertoires of VH or VL genes amplified from the mRNA of spleen cells of an immunized mouse are therefore greatly enriched in V genes encoding part of an antigen binding site (91).

In random combinatorial libraries (48), the VH and VL gene repertoires are combined at random, and the original combinations of the immune lymphocyte are destroyed. Nevertheless, if the V gene repertoires are derived from the mRNA of lymphocytes after immunization, antigen binding fragments are created at low frequency, at best <1/500 (92), and more usually <1/5000 (93, 94). The power of phage selection allows

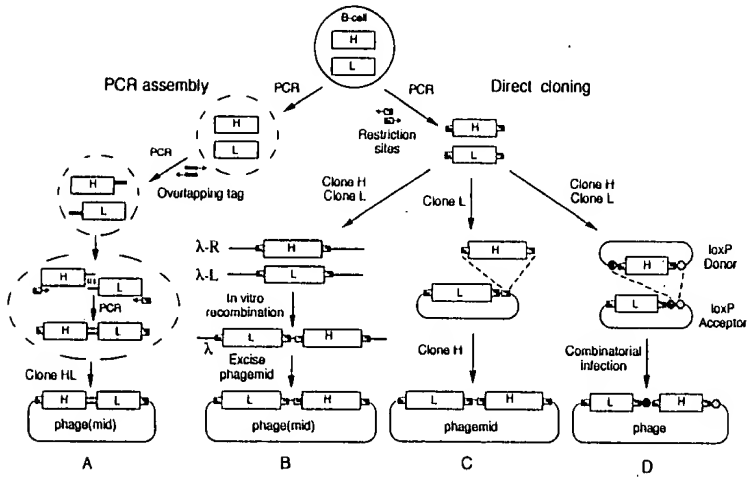

Figure 5 Linking the V genes together. (A) PCR assembly allows a one-step cloning of heavy (H) and light (L) DNA in scrambled pairings (34, 37), or original pairings if "in-cell" (89). Alternatively (B, C, D) heavy (H) and light (L) chain DNA is cloned separately and combined by in vitro recombination (B) (87), or combinatorial infection (D) (88), or cloned sequentially (C) (16).

many of these fragments to be isolated and characterized. For example, a repertoire of antibody fragments was assembled for phage display from the mRNA of mouse splenocytes after immunization with the hapten phenyloxazolone (phOx). The VH and Vκ genes encoding a range of fragments were found to be similar to those of hybridomas of the phOx response, but in general the pairings were not (37, 95).

Furthermore, as suggested by λ phage combinatorial libraries (93, 94), the pairings were promiscuous, that is, the same light chain could be found with different heavy chains, and vice versa. By "shuffling" a promiscuous heavy chain with the repertoire of light chains, a further range of partners were found for binding to phOx (37). Combinatorial repertoires from immunized sources therefore appear to be dominated by "artificial" pairings, as predicted (4). Although original pairings are likely to be present in large random combinatorial libraries, it is impossible to distinguish original from artificial pairings. However, it may be possible to determine these pairings by first linking the VH and VL genes within the lymphocyte (89)

Nevertheless, the artificial pairings from phage display libraries and, enriched by immunization, can provide antibody fragments with good affinities. For example, an antibody fragment isolated from the phOx

response (as above) had a binding affinity of 10^8 M^{-1} for hapten, with every prospect that higher affinity antibodies were present in the repertoire (37). This compares with typical affinities of 10^8 M^{-1} for secondary phOx antibodies from hybridomas (62, 96), and with affinities of 7.5×10^6–4×10^8 M^{-1} for hybridomas isolated from the same immunized spleen (95).

Antibody fragments have also been isolated from immunized humans with binding activities against several viral antigens, for example, HIV gp120 (54, 97, 98), respiratory syncytial virus (RSV) (99), and hepatitis B virus (100). The fragments against HIV and RSV were capable of neutralizing virus infection (97, 99). Furthermore, specificities against herpes simplex virus, human cytomegalovirus, varicella zoster virus, rubella, RSV, and HIV were derived from the same V gene repertoire from a patient immune to these pathogens (101). Extensive chain promiscuity has also been seen for human antibody fragments derived from combinatorial libraries directed against HIV gp 120: it was argued that the heavy chains must have arisen from antigen-specific clones in vivo (98).

By-Passing Immunization

As the display and selection of antibodies on phage mimicks immune selection, it should be possible to isolate antibody fragments of any required specificity directly from a single phage repertoire of sufficient size and diversity. Importantly, it should provide antibody specificities directed against self-antigens that are difficult to raise by immunization, owing to tolerance mechanisms.

NATURAL REPERTOIRES A diverse source of rearranged V genes was provided by human peripheral blood lymphocytes (PBLs), using "family-based" PCR primers to amplify each of the human VH, Vκ, and Vλ families (80). The repertoires of VH and VL genes were combined at random, as this should destroy the original combinations and specificities of the PBLs and generate new specificities (34).

From this library, it was possible to isolate phage with binding activities against many different antigens. For example, antibodies were isolated against the foreign antigens bovine serum albumin (BSA), turkey egg lysozyme, the hapten phOx (34), and bovine thyroglobulin (57), and against the human self-antigens tumor necrosis factor α (TNFα), thyroglobulin, a monoclonal antibody, carcinoembryonic antigen (CEA), mucin and CD4 (57). Antibody fragments against the monoclonal antibody mapped to both variable and constant regions (57). Antibodies were also isolated against the human blood group antigens of the ABO and I blood group systems (B and HI), of the Rh system (D and E), and of the Kell system (Kpb) (35). For the anti-blood groups, the selections were

undertaken by binding the phage to red blood cells; the anti-E phage was only selected after first preabsorbing the phage library with red cells lacking this antigen.

The antibodies from the library were shown to be highly specific by screening for binding to a panel of other antigens (34, 57). Specificity was also demonstrated by the staining of kidney sections with the anti-B: the only cells stained were the endothelial cells bearing the blood group B antigen (35). The affinities of the antibodies were typical of a primary immune response, in the range 10^5 M^{-1}–10^7 M^{-1}, but dimerization of the scFv fragments led to improved avidities (57). Antibody fragments were also derived from V genes prepared from unimmunized rodent bone marrow. However, the library was selected only against the hapten progesterone, the binding affinities were poor (apparently 10^4–10^5 M^{-1} by competition ELISA), and the fragments cross-reacted with another protein (56).

Although a range of anti-self specificities can be derived from a "single pot" library from "natural" rearranged V genes, it is impossible to prove that one or another of the antibody chains was not derived from B cells with self-specificity. Moreover, in most cases the sequences of both chains were somatically mutated, suggesting that the chains were derived from an antigen-driven process (35, 57); indeed and for the anti-blood group B specificities, anti-B could be detected in the donor antiserum (35).

SYNTHETIC REPERTOIRES Synthetic V gene repertoires can also be built from cloned human VH-gene segments. A repertoire (2×10^7 clones) was first constructed using a short H3 loop of five or eight random residues with each of 49 segments, and combined with a fixed light chain. Antibodies of high specificity were selected against two haptens, phOx and NIP (with affinities of up to 10^6 M^{-1}) and human TNF-α, but not against three other (protein) antigens (47). However, by adding a range of H3 loops of different lengths, up to 12 residues, a single library was created from which a range of more than 20 binding specificities could be selected, including against haptens; the foreign antigens lysozyme, keyhole limpet haemocyanin, streptavidin, and immunoglobulin binding protein (BIP); and the self-antigens the oncogene protein rhombotin and the tumor suppressor protein p53. The epitope of an antibody binding to p53 was mapped and found to be new. The antibodies appeared to be specific and could be used as reagents for immunofluorescence staining of p53 in the nuclei of cells, and for Western blotting of cell lysates for BIP (A Nissim, unpublished). This also illustrates that antibodies can be made against intracellular antigens, and in particular those of the lumen of the endoplasmic reticulum.

Other synthetic libraries have been built from the framework of a single antibody. By randomizing the H3 loop a single binding specificity was selected against FITC (affinity 10^7 M^{-1}) (82); by randomizing the sequences of the L1, L3, H2, and H3 loops, a single binding specificity was selected against insulin-like growth factor (but not against CD4 or tissue plasminogen activator) (102). There clearly has to be sufficient structural diversity to make a working "single-pot" library.

MAKING HIGH AFFINITY ANTIBODIES

Mutation

For most purposes, antibodies must bind their antigen tightly. In the immune system, strong binding can be built from multiple weak interactions, as illustrated by the interactions of IgM with multivalent antigens such as virus. However, the higher affinity antibodies are made after repeated rounds of immunization, arising either as mutants of a primary response antibody, or as entirely new antibodies (repertoire: such antibodies may arise by somatic mutation of very low affinity antibodies (96). The increase in binding affinity of primary response antibodies is sometimes modest, with anti-NP hybridomas showing a five-fold improvement in affinity (103), or large, with anti-phOx hybridomas showing improvements of 100-fold (62). Site-directed mutagenesis of an anti-p-azophenylarsonate antibody also suggests that somatic mutation at a few sites can together contribute factors of >200 to binding affinity (104).

In phages, antibody fragments can be designed with higher binding avidities, for example, as single chain dimers (57) or "diabodies" (58). Presumably other multimeric fragments could be designed to mimic IgM. Furthermore, mutation can be introduced at random in vitro (36, 56) by using error prone polymerase (105), or in vivo by use of mutator strains of bacteria (106, 107), and the phage can be selected for higher affinities. However, the affinities of antibody fragments against a hapten and a protein antigen could be improved only a modest four-fold to 10^8 M^{-1} and 10^9 M^{-1}, respectively, using a single round of random mutation followed by multiple rounds of selection (36; RE Hawkins, SJ Russell, unpublished data). To make higher affinity mutants, it might be desirable to increase the frequency of random mutation or to combine rounds of mutation and selection, for example, by growing phage in bacterial mutator strains. Alternatively, it might be desirable to start with lower affinity antibodies (as may occur in repertoire shift), in the event that a higher affinity binding site is trapped at a local optimum and incapable of further affinity maturation (108).

Phage display appears to have potential advantages over the immune system for the creation of secondary (mutated) repertoires. Firstly, the size

of the secondary repertoires can be much larger than in immune systems. Secondly, random mutation can be focussed to the antigen binding loops or outside, for example, at framework residues that influence loop conformation (63). Indeed, mutations outside the contact surface with antigen can often have profound effects on binding affinity (104, 109, 112).

Chain Shuffling

In the immune system, somatic mutation of a selected pair of VH and VL domains appears to be the only mechanism for making structural variation of a selected antigen binding site. However, random combinatorial repertoires contain immense untapped diversity that can be mobilized by chain shuffling.

Chain shuffling was first used to analyze the promiscuity of VH and VL pairings in repertoires from immunized mice (37, 110). It was then used for the affinity maturation of a human antibody fragment (affinity 3×10^6 M^{-1}) for phOx isolated from a V gene repertoire. The VH gene was paired with VL genes from the original repertoire, and the new (light chain shuffled) repertoire was displayed on phage. A light chain partner was isolated that conferred improved binding affinity (6×10^7 M^{-1}). Likewise the new VL gene was paired with the original repertoire of VH genes, (but now combined with the H3 loop of the original VH gene), and after selection a fragment was isolated with a further improved affinity (10^9 M^{-1}). Indeed the affinities of the original and shuffled fragments are similar to those of mouse hybridomas of the primary and later responses to the same hapten.

In the high affinity fragment, both domains were derived from the same germline VH and VL genes as the parent, but with different patterns of mutations. The 20-residue changes suggest that large changes in affinity (500-fold here) might require many random mutations (38).

Chain shuffling can therefore be used to tap the somatically mutated V genes and make higher affinity binding sites. However, chain shuffling can also be used for more extensive diversification. For example, the heavy and light chains of mouse monoclonal antibodies against the hapten phOx (M Figini, unpublished) and human TNFα (H Hoogenboom, L Jespers, unpublished data) were sequentially replaced to create entirely human antibodies of the same specificity, a process termed *epitope imprinted selection*.

Large Repertoires

Theoretical studies have suggested, not surprisingly, that the larger the library, the greater the chance of finding antibodies that bind to any given epitope, and the higher the affinity (111). However, the limiting factor in making large primary libraries is the efficiency of introduction of plasmid or phage DNA into bacteria. In practice, this limits the library size to 10^7–10^9 clones, even taking advantage of λ phage vectors with excisable filamentous phage replicons (87).

In principle, a simple way of increasing library size would be to generate more of the possible chain combinations. This has prompted a new approach—combinatorial infection (88). For example, if 10^5 different light chains were cloned for display as a Fab-pIII fusion in a phage vector, and then the phage used to infect $> 10^{12}$ bacteria harboring a library of 10^7 different heavy chains in a plasmid, this could create 10^{12} possible Fab fragments (15). If the two chains were recombined efficiently in vivo onto the same phage replicon by use of loxP sites (88), this would create a phage library with huge diversity. Indeed, it appears that such huge "teraphage" libraries can be created (88; AD Griffiths, P Waterhouse, unpublished), and this should allow high affinity antibodies to be isolated directly and indeed might also facilitate any chain shuffling required for further affinity improvements.

CONCLUSION

Phage display should facilitate the construction of human antibodies of therapeutic value and of research reagents. Libraries have been constructed that take advantage of immunization, or by-pass it, leading to antibodies with good binding affinities (10^8–10^9 M^{-1}) and high specificity against foreign and self-antigens. Targets have included viral coat proteins, BIP from the lumen of the endoplasmic reticulum, and surface markers of lymphocytes (T cell receptor and CD4), tumor cells (CEA and mucin) and red blood cells (B, D, E, I and Kell). The antibodies have been used to neutralize virus, to stain cells, and for Western blots.

There is clearly a future for "single pot" libraries, as the same library can be selected with a range of different antigens, and without the need for immunization of animals. The affinities of the antibodies isolated should improve as new technologies are used to increase the size and diversity of libraries. Indeed, the availability of the cloned human VH, Vκ, and Vλ gene segments, and knowledge about the structures they encode, should allow the design of maximum structural diversity in primary repertoires. It should also allow the creation of premutated genes for use in making secondary repertoires, in which mutations are focussed at the antigen contacts or at sites likely to modulate the contacts.

There may also be a future for "designer" libraries. As the potential antibody diversity is probably too large to be tapped in a single phage library, it may be advantageous to build libraries that are shaped for

complementarity to a defined antigen. As phage display can not only exploit the principles of immune selection, but also cannibalize and improve on the antibody building blocks, it should increasingly be capable of outperforming natural immune systems in making antibodies.

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IMMUNOBIOLOGY

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

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CURRENT
BIOLOGY
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Current Biology Ltd
London, San Francisco and Philadelphia

Garland Publishing Inc
New York and London

Principal text editor: Miranda Robertson
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Principal designer and illustrator: Celia Welcomme
Designer: Sylvia Purnell
Assistant Illustrator: Matthew McClements
Production: Rebecca Spencer
Graphics software support: Gary Brown
Proofreader: Melanie Paton
Indexer: Nina Boyd
Photo research: Doug McGaughy, Tamsin Newmark

Distributors
Inside North America: Garland Publishing Inc., 717 Fifth Avenue, New York, NY 10022, USA.
Inside Japan: Nankodo Co. Ltd., 42-6, Hongo 3-Chome, Bunkyo-ku, Tokyo 113, Japan.
Outside North America and Japan: Blackwell Scientific Publications, Osney Mead, Oxford OX2 0EL. Orders to: Marston Book Services Ltd, PO Box 87, Oxford OX2 0DT, UK.
Australia: Blackwell Scientific Publications Pty Ltd., 54 University Street, Carlton, Victoria 3053.

ISBN 0-8153-1497-3 (hardcover) Garland
ISBN 0-8153-1691-7 (paperback) Garland
ISBN 0-86542-811-5 (paperback) Blackwell

A catalog record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data
Janeway, Charles.
Immunobiology: the immune system in health and disease/ Charles A. Janeway, Jr., Paul Travers.
p. cm.
Includes bibliographical references and index.
ISBN 0-8153-1497-3 (hardcover). ISBN 0-8153-1691-7 (pbk.).
1. Immune System. 2. Immunity. I. Travers, Paul, 1956- . II. Title
[DNLM: 1. Immune System--physiology. 2. Immune System--physiopathology. 3. Immunity--physiology. 4. Immunotherapy. QW 504 1994]
QR181.J37 1994
616. 07'9--dc20
DNLM/DLC
for Library of Congress 94-11058
CIP

This book was produced using Ventura Publisher 4.1 and CorelDraw 3.0.

Printed in Hong Kong by Paramount Printing Co. Ltd.

Published by Current Biology Ltd., Middlesex House, 34-42 Cleveland Street, London W1P 5FB, UK and Garland Publishing Inc., 717 Fifth Avenue, New York, NY 10022, USA.

2-11 Monoclonal antibodies have a homogeneous structure and can be produced by cell fusion or by genetic engineering.

Even antibodies specific for the same small defined antigenic determinant are heterogeneous in structure, as shown by their differing isoelectric points, making detailed chemical analysis of such antibodies impossible. In order to examine a homogeneous preparation of antibody, biochemists first analyzed proteins produced by patients with multiple myeloma, a common tumor of plasma cells. It was known that antibodies are normally produced by plasma cells, and as this disease is associated with the presence of large amounts of a homogeneous gamma globulin called a myeloma protein in the patient's serum, it seemed likely that myeloma proteins would serve as models for normal antibody molecules. Thus, much of the early knowledge of antibody structure came from studies on myeloma proteins. However, these proteins had one major limitation for such studies; the antigen specificity of most myeloma proteins was not known.

This problem was solved by Georges Köhler and César Milstein, who devised a technique for producing a homogeneous population of antibodies of known antigen specificity. They did this by fusing spleen cells from an immunized mouse to cells of a mouse myeloma to produce hybrid cells that both proliferated indefinitely and secreted antibody specific for the antigen used to immunize the spleen cell donor. The spleen cell provides the ability to make specific antibody, while the myeloma cell provides the ability to grow indefinitely in culture and secrete immunoglobulin continuously. By using a myeloma cell partner that produces no antibody proteins itself, the antibody produced by the hybrid cells comes only from the immune spleen cell partner. After fusion, the hybrid cells are selected using drugs that kill the myeloma parental cell, while the unfused parental spleen cells have a limited lifespan and soon die, so that only hybrid myeloma cell lines or **hybridomas** survive. Those hybridomas producing antibody of the desired specificity are then identified and cloned by regrowing the cultures from single cells. Since each hybridoma is a **clone** derived from a single B cell, all the antibody molecules it produces are identical in structure, including their antigen-binding site and isotype. Such antibodies are therefore called **monoclonal antibodies** (Fig. 2.19). This technology has revolutionized the use of antibodies by providing a limitless supply of antibody of a single and known specificity and a homogeneous structure. Monoclonal antibodies are now used in most serological assays, as diagnostic probes, and as therapeutic agents.

Recently, a novel technique for producing antibody-like molecules has been introduced. Gene segments encoding the antigen-binding variable or V domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophage. A collection of recombinant phage, each displaying a different antigen-binding domain on its surface, is known as a **phage display library**. In much the same way that antibodies specific for a particular antigen can be isolated from a complex mixture by affinity chromatography (see Section 2-7), phage expressing antigen-binding domains specific for a particular antigen can be isolated by selecting the phage in the library for binding to that antigen. The phage particles that bind are recovered and used to infect fresh bacteria. Each phage isolated

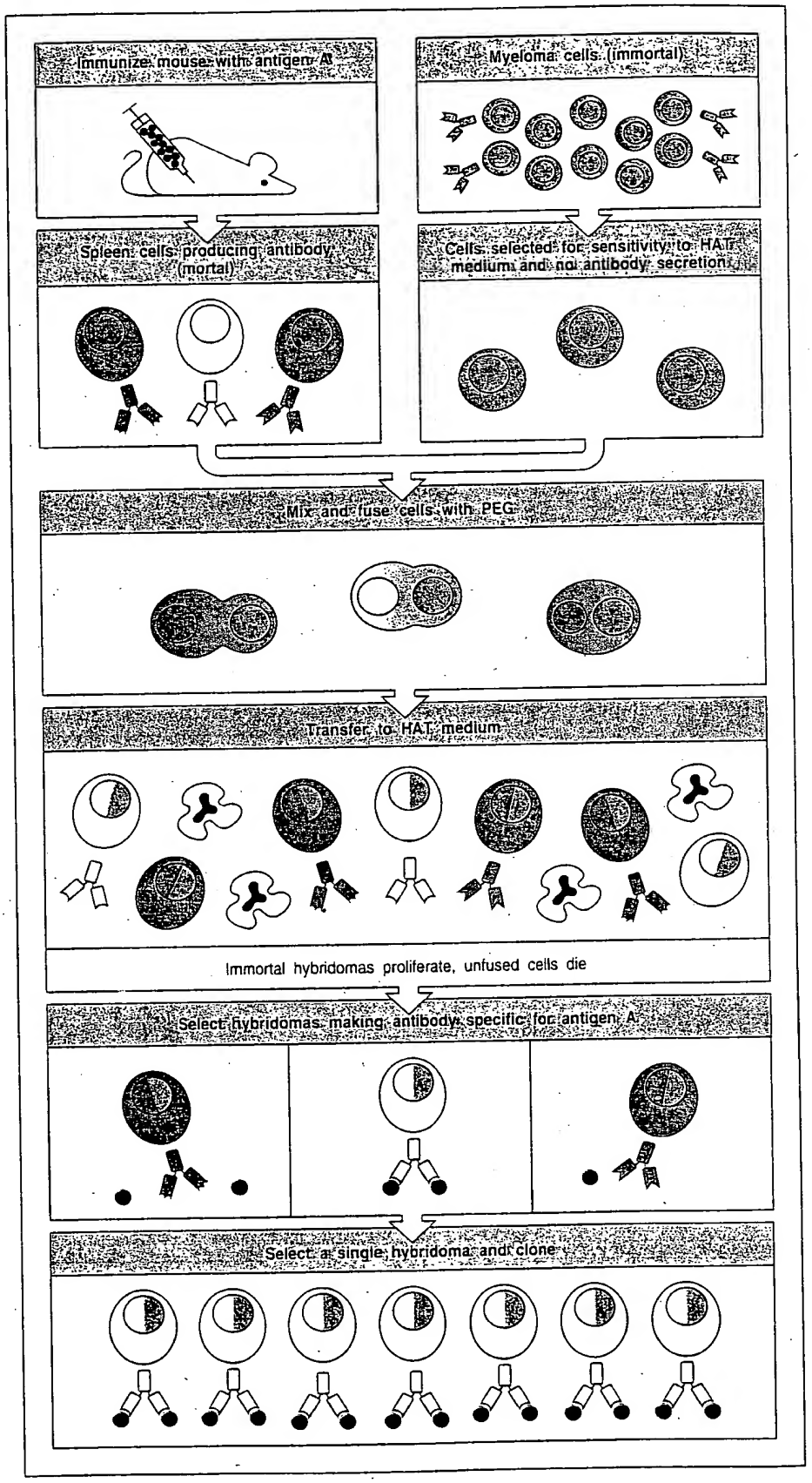

Fig. 2.19 Th production of monoclonal antibodies. Mice are immunized with antigen A and given an intravenous booster immunization three days before they are killed in order to produce a large population of spleen cells secreting specific antibody. Spleen cells die after a few days in culture. In order to produce a continuous source of antibody they are fused with immortal myeloma cells using polyethylene glycol (PEG) to produce a hybrid cell line called a hybridoma. The myeloma cells are selected beforehand to ensure that they are not secreting antibody themselves and that they are sensitive to the HAT medium that is used to select hybrid cells because they lack the enzyme hypoxanthine:guanosine phosphoribosyl transferase (HGPRT). The HGPRT gene contributed by the spleen cell allows hybrid cells to survive in the HAT medium, and only hybrid cells can grow continuously in culture because of the malignant potential contributed by the myeloma cells. Therefore, unfused myeloma cells and unfused spleen cells die in the HAT medium, as shown here by cells with dark, irregular nuclei. Individual hybridomas are then screened for antibody production, and cells that make antibody of the desired specificity are cloned by growing them up from a single antibody-producing cell. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. As each hybridoma is descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule, which is called a monoclonal antibody.

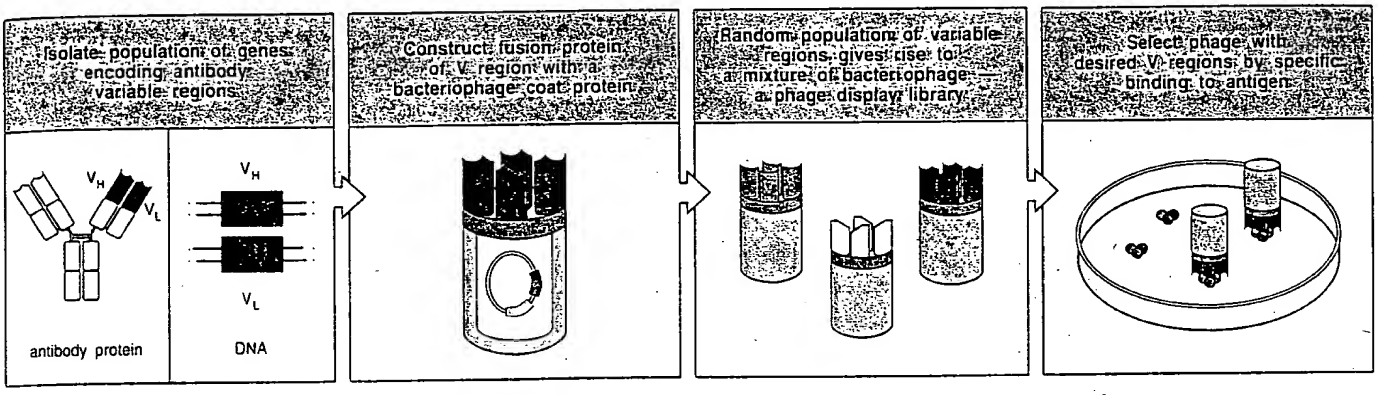

Fig. 2.20 The production of antibodies by genetic engineering. Short primers to consensus sequences in heavy- and light-chain variable or V regions of immunoglobulin genes are used to generate a library of heavy- and light-chain V-region cDNAs by the polymerase chain reaction (see Fig. 2.50) using spleen mRNA as the starting material. These heavy- and light-chain V-region genes are cloned randomly into a filamentous phage such that each phage expresses one heavy- and one light-chain V region as a surface fusion protein with antibody-like properties. The resulting phage display library is expanded in bacteria, and the phage are then bound to a surface coated with antigen. The unbound phage are washed away, while the bound phage are recovered and again bound to antigen. After a few cycles, only specific, high-affinity antigen-binding phage are left. These can be used like antibody molecules, or their V genes can be recovered and engineered into antibody genes to produce genetically engineered antibody molecules (not shown). This technology may replace the hybridoma technology for producing monoclonal antibodies and has the advantage that any species can be used as the source of the initial mRNA.

in this way will produce a monoclonal antigen-binding particle analogous to a monoclonal antibody (Fig. 2.20). The genes encoding the antigen-binding site, which are unique to each phage, can then be recovered from the phage DNA and used to construct genes for a complete antibody molecule by joining them to gene segments that encode the invariant parts of an antibody. When these reconstructed antibody genes are introduced into a suitable host cell line, such as the non-antibody producing myeloma cells used for hybridomas, the transfected cells secrete antibodies with all the desirable characteristics of monoclonal antibodies produced from hybridomas. This technique may ultimately replace the traditional route of cell fusion for production of monoclonal antibodies.

2-12 The affinity of an antibody can be determined directly by binding to small ligands.

The **affinity** of an antibody is the strength of binding of a monovalent ligand to a single antigen-binding site. The affinity of an antibody that binds small antigens such as haptens that can diffuse freely across a dialysis membrane can be determined directly by the technique of **equilibrium dialysis**. A known amount of antibody, whose molecules are too large to cross a dialysis membrane, is placed in a dialysis bag and offered varying amounts of antigen. Molecules of antigen that bind to the antibody are no longer free to diffuse across the dialysis membrane, so only the unbound molecules of antigen equilibrate across it. By measuring the concentration of antigen inside the bag and in the surrounding fluid, one can determine the amount of the antigen that is bound as well as the amount that is free when equilibrium has been achieved. Given that the amount of antibody present is known, the affinity of the antibody and the number of specific binding sites for the antigen per molecule of antibody can be determined from this information. The data is usually analyzed by **Scatchard**

1: Immunology 1986 May;58(1):157-60

Related Articles, Links

Production of human monoclonal IgG and IgM antibodies with anti-D (rhesus) specificity using heterohybridomas.

Thompson KM, Melamed MD, Eagle K, Gorick BD, Gibson T, Holburn AM, Hughes-Jones NC.

Heterohybridomas secreting human IgM and IgG anti-D antibodies of the rhesus blood group system have been established by fusion of EBV-transformed anti-D secreting cells with the mouse myeloma cells X63-Ag8.653. Both classes of antibody reacted with all Rh-positive cells, some Du cells but not with Rh-negative or DB cells. Concentrations of both antibodies reached between 25 micrograms/ml and 50 micrograms/ml in the culture supernatants. The cell lines have been maintained in culture for 14 months and have been shown to be suitable for large-scale production of antibody.

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1: Acta Haematol 1988;79(3):137-9

Related Articles, Links

Generation of multiple monoclonal antibodies for diagnostic use from a single hybridoma fusion.

Matsuo S, Penneys NS, Ziegels-Weissman J, Nadji M.

Department of Dermatology, Asahikawa Medical College, Japan.

BALB/c mice were exposed simultaneously to three nonrelated immunogens, myelin basic protein, uridyl-galactosyl transferase, and tissue obtained from a formalin-fixed, paraffin-embedded block containing a pilomatrixoma. Standard hybridoma techniques were used and antibody generation assayed using an unlabelled antibody biotin-avidin method with sections of human cerebellum, liver, and pilomatrixoma as the substrates. Using the above assay, clones were selected that secreted antibodies with selective specificities for each of the immunogens. By ELISA assay, the monoclonal antibodies reacting with cerebellum also reacted to the myelin basic protein preparation used as immunogen and the monoclonal antibody reacting to hepatocytes bound to the preparation of uridyl-galactosyl transferase used as immunogen. Our data suggests that the generation of monoclonal antibodies with a variety of diagnostic applications can be obtained from a single fusion following immunization with multiple nonrelated antigens, requiring considerably less laboratory cost and effort than would be required to obtain similar monoclonal antibodies in separate fusions.

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1: Immunol Lett 1992 Aug;33(3):217-22

Construction of a quadroma to alpha-endorphin/horseradish peroxidase using an actinomycin D-resistant mouse myeloma cell line.

Massino YuS, Kizim EA, Dergunova NN, Vostrikov VM, Dmitriev AD.

Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, Moscow.

A hybrid hybridoma (quadroma), secreting antibodies with double specificity to alpha-endorphin (alpha-EP) and horseradish peroxidase (HRP), has been produced. The bispecific antibodies constituted about 28-29% of all immunologically active IgG, produced by quadroma. The quadroma was isolated by fusion of two mouse hybridomas (anti-HRP and anti-alpha-EP) with distinct phenotypes: double mutant AMDR/HAT(S), and wild type (AMDS/HATR). A novel strategy for the construction of a double-mutant was applied, based on the use of an actinomycin D-resistant (AMDR) mouse myeloma for initiation of one of the parental hybridomas.

PMID: 1358818 [PubMed - indexed for MEDLINE]